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br regulates the expression of the ecdysone biosynthesis gene *npc1*

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ABSTRACT

The growth and metamorphosis of insects are regulated by ecdysteroid hormones produced in the ring gland. Ecdysone biosynthesis-related genes are both highly and specifically expressed in the ring gland. However, the intrinsic regulation of ecdysone biosynthesis has received little attention. Here we used the *Drosophila npc1* gene to study the mechanism of ring gland-specific gene expression. *npc1* is important for sterol trafficking in the ring gland during ecdysone biosynthesis. We have identified a conserved ring gland-specific *cis*-regulatory element (RSE) in the *npc1* promoter using promoter fusion reporter analysis. Furthermore, genetic loss-of-function analysis and *in vitro* electrophoretic mobility shift assays revealed that the ecdysone early response gene *broad complex* (*br*) is a vital factor in the positive regulation of *npc1* ring gland expression. Moreover, *br* also affects the ring gland expression of many other ecdysone biosynthetic genes as well as *torso* and *InR*, two key factors in the regulation of ecdysone biosynthesis. These results imply that ecdysone could potentially act through its early response gene *br* to achieve positive feedback regulation of ecdysone biosynthesis during development.

Introduction

The postembryonic development of insects is regulated by a neuroendocrine complex consisting of two main parts: secretory neurons of the central nervous system (CNS) and endocrine glands including the prothoracic gland (PG), the corpora allata (CA), and the corpus cardiacum (CC) (Andres and Thummel, 1992; Siegmund and Korge, 2001). The PG produces and secretes the steroid hormone ecdysone which is converted to 20-hydroxyecdysone (20E) in peripheral tissues. 20E is the principal molting hormone of arthropods and regulates insect growth, molting, and metamorphosis (Gilbert and Warren, 2005). Ecdysone and 20E can bind to the nuclear ecdysone receptor (EcR) and switch on the transcription of a set of ecdysone early response genes, including *broad complex* (*br*), *E74*, and *E75* (Basso et al., 2006; Thummel, 1990). These genes subsequently turn on the expression of late response genes to accomplish insect development. Therefore, understanding the function and the regulation of the ecdysone signaling pathway is critical to insect biology and pest control.

Regulation of the ecdysone signaling pathway could take place in signal-producing cells where ecdysone is biosynthesized or in signal receiving cells where the EcR is situated. Ecdysone biosynthesis has been studied extensively in the model organism, *Drosophila melanogaster* (Gilbert and Warren, 2005). In higher dipterans, including *Drosophila*, the larval PG, CA, and CC are fused into a single compound structure, named the ring gland. Since the PG is the largest part of the ring gland, it is often referred to simply as the ring gland. The production of ecdysone in the *Drosophila* ring gland is a complex process, involving many biosynthetic enzymes as well as the cellular regulatory machinery (Andrews et al., 2002; Gilbert and Warren, 2005). Many ecdysone-deficient mutants have been identified through numerous genetic screens. The most obvious phenotype of ecdysone deficiency is arrest of larval development. In addition, most of the mutants can be partially rescued by supply of ectopic ecdysone. Of the known ecdysone-deficient mutants, *dare*, *dib*, *neverland*, *phm*, *sad*, *shd*, and *spo* encode ecdysone biosynthetic enzymes (Chavez et al., 2000; Freeman et al., 1999; Gilbert, 2004; Namiki et al., 2005; Ono et al., 2006; Petryk et al., 2003; Warren et al., 2004; Yoshiyama et al., 2006); *ecd1* encodes a novel conserved protein and its human homology may be involved in transcriptional regulation (Gaziova et al., 2004); and *mld*, *woc*, and *ftz-F1* encode transcriptional regulators (Gaziova et al., 2004; Neubueser et al., 2005; Parvy et al., 2005; Warren et al., 2001).

It is known that the PTTH and its receptor Torso are the upstream triggers for ecdysone biosynthesis (McBrayer et al., 2007; Rewitz et al., 2009). In addition, it has been shown that insulin signal pathway can increase ecdysone levels in the ring gland (Colombani et al., 2005). During development, ecdysone biosynthesis is tightly regulated with the level of ecdysone peaking before molting. Not surprisingly, most genes participating in ecdysone biosynthesis are highly and specifically expressed in the ring gland. However, the spatial regulation of the expression has been barely explored. In addition, to

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achieve such a precise temporal pattern of ecdysone biosynthesis, the expression of ecdysone biosynthesis-related genes must be coordinately regulated. Currently, little is known about the regulation mechanisms involved. Although several ring gland-specific transcription regulators have previously been identified, their respective *cis*-regulatory elements are not known, and very few targets of these transcription regulators have been found (Roth et al., 2004).

As a sterol auxotrophic organism, *Drosophila* synthesizes ecdysone from dietary sterols. Therefore, dietary sterol intake and transport may play an important role in ecdysone biosynthesis (Huang et al., 2008). We previously reported that mutants in *Niemann-Pick type C1* (*npc1*) or *C2* (*npc2*) genes are defective in ecdysone biosynthesis (Huang et al., 2005, 2007). NPC disease is a fatal autosomal recessive neurodegenerative disorder characterized by the inappropriate accumulation of unesterified cholesterol in aberrant organelles (Carstea et al., 1997). In *Drosophila*, *npc1* is normally highly expressed in the ecdysone-producing organ, ring gland. Expression of *npc1* in the ring gland alone can rescue the *npc1* mutant larval arrest phenotype, indicating that *npc1* ring gland expression is important for fly development (Huang et al., 2005). In this study, we performed *npc1* promoter analysis and identified a ring gland-specific *cis*-element, RSE. In addition, we found that the well-known ecdysone early response gene *br* could positively regulate the transcription of *npc1* and a set of ecdysone biosynthetic genes. In addition, the transcriptions of *torso* and insulin receptor gene *InR* are all reduced in the ring glands of *br* mutants and ring gland-specific *br* RNAi animals. *br* is an important transcription regulator for many genes during organ development in response to ecdysone signal (Fletcher and Thummel, 1995). Thus, ecdysone could potentially act through its early response gene to achieve positive feedback regulation of ecdysone biosynthesis during the rapid rise in the levels of ecdysone.

Materials and methods

Fly stocks and genetics

Flies were cultured on standard cornmeal food at 25 °C. The *woc* and *mld* strains were generous gifts from Lawrence Gilbert; the *ftz-f1* strains were obtained from Hitoshi Ueda; the *hsp-br Z1*, *Z2*, *Z3*, and *Z4* transgenes were kindly provided by Lynn Riddiford; and the $npc1^{27A}$ strain was kindly provided by Leo Pallanck. Other stocks were obtained from the Bloomington Stock Center. Transgenic stocks were generated by standard methods.

ftz-f1 mutant clones were generated by mitotic recombination using the FLP/FRT system in *hs-flp*/+;*npc1-Gal4*>*UAS-myr-RFP*/+; $ftz\text{-}f1^{ex7}$ *FRT2A*/*his-GFP FRT2A* embryos. Embryos between 6.5 and 7.5 h after egg-laying were heat-shock treated at 38 °C for 1 h to induce flipase expression. Larvae were dissected at the wandering stage of the third instar. FRT-mediated recombination events were identified by loss of nuclear GFP expression.

Molecular cloning

Full-length sequences and truncations of the *Drosophila npc1* promoter were amplified from genomic DNA by PCR and were cloned into the *Stu*I–*Bam*HI sites of the *pC3G4* vector or the *pPT-GAL* vector, which contains an *hsp70* minimal promoter. The BR-Z4 binding site deletion and point mutation fragments were amplified by fusion PCR using the 220-bp RSE as a template and were then cloned into the *Stu*I–*Bam*HI sites of *pPT-GAL*. The full-length BR-Z4 and Z4 DNA binding domain (amino acid 524 to the stop codon) were amplified from BR-Z4 cDNA clone *LP12157*. Full-length BR-Z4 was in frame-subcloned into the *Eco*RI–*Sal*I sites of *pGEX-4T-1*. The Z4 DNA-binding domain was subcloned into the *Eco*RI–*Sal*I sites of *pGEX-4T-2*. The GST fusion protein was purified by Glutathione Sepharose 4B (GE healthcare Bio-science AB) and then stored in aliquots at −70 °C.

Quantitative RT–PCR and in situ hybridization

For quantitative RT–PCR (qRT–PCR) analysis total RNA was extracted from ring glands using a TRIzol kit (Invitrogen). Isolated total RNA was reverse-transcribed into cDNA using M-MLV reverse transcriptase (Invitrogen). All subsequent qRT–PCRs were performed on an ABI PRISM 7900HT real-time cycler (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems). The primer sequences are listed in Supplement Table 1.

For *in situ* hybridization, ring gland complexes attached to the brain of third instar larvae were dissected in phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA). Embryos were collected 10 h after egg-laying. After removing the chorion and vitelline membrane, these embryos were fixed in 4% PFA. Hybridization procedures and probes used were the same as previously described (Huang et al., 2005).

Nuclear extract preparation and electrophoretic mobility shift assay (EMSA)

Embryo or larval nuclear extracts were prepared with protocols described before (Pazin, 2000). The protein concentrations of nuclear extracts were measured using a BCA kit and the extracts were stored in aliquots at −70 °C. EMSA was carried out with a DIG Gel Shift Kit, second generation (Roche). The incubation time for the probe and protein mixture was 15 min. The amounts of proteins and RSE core probes used in each experiment are indicated in the figure legends. The RSE core probe was six repeats of the BR-Z4 binding site: TGTTTAC. The mutated probe was six repeats of the following sequence: TGCCCAC.

Results

npc1 transcription is not regulated by woc, mld, ecd1, and ftz-f1

To identify potential transcriptional regulators for ring gland-specific expression of *npc1*, we first tested several known candidate genes which were reported to be important for ecdysone biosynthesis and likely encode transcriptional regulators, including *woc*, *mld*, *ecd1*, and *ftz-f1*. *woc*, *mld*, and *ecd1* mutants are not embryonic lethal, so we examined the expression of *npc1* by *in situ* hybridization in late-stage embryos or young larvae. We found that the ring glands of these mutants were strongly marked by the *npc1* anti-sense probe, indicating that ring gland-specific expression of *npc1* is not affected in these mutants (Figs. 1A–D). *ftz-f1* mutants are early embryonic lethal. We used mosaic analysis combined with the *UAS-Gal4* system to detect *npc1* ring gland expression. A 2.3-kb *npc1* promoter *Gal4* fusion was used to drive *UAS-myr-RFP* expression specifically in the ring glands (see below). We reasoned that if *ftz-f1* regulates ring gland-specific expression of *npc1*, then *npc1-Gal4* would not be activated to drive *UAS-myr-RFP* expression in *ftz-f1* mutant clones. In the wild type, the myr-RFP signal highlights the membrane boundary of ring gland cells. While in *ftz-f1* clones, although more myr-RFP is localized in cytoplasm than in the plasma membrane, we found that the RFP signal persisted (Figs. 1E and F), indicating that the *ftz-f1* mutation does not affect *npc1* expression. Instead, *ftz-f1* may regulate the expression of genes involved in protein intracellular trafficking. Therefore, in the ring gland, *npc1* transcription is unlikely to be regulated by these four known ecdysone pathway-related transcription regulators.

A ring gland-specific DNA element (RSE) is required for npc1 expression

We then defined the *cis*-regulatory elements in the *npc1* promoter and identified the *trans*-regulatory factors that bind to the *cis* elements. We first generated a promoter–*Gal4* fusion transgene

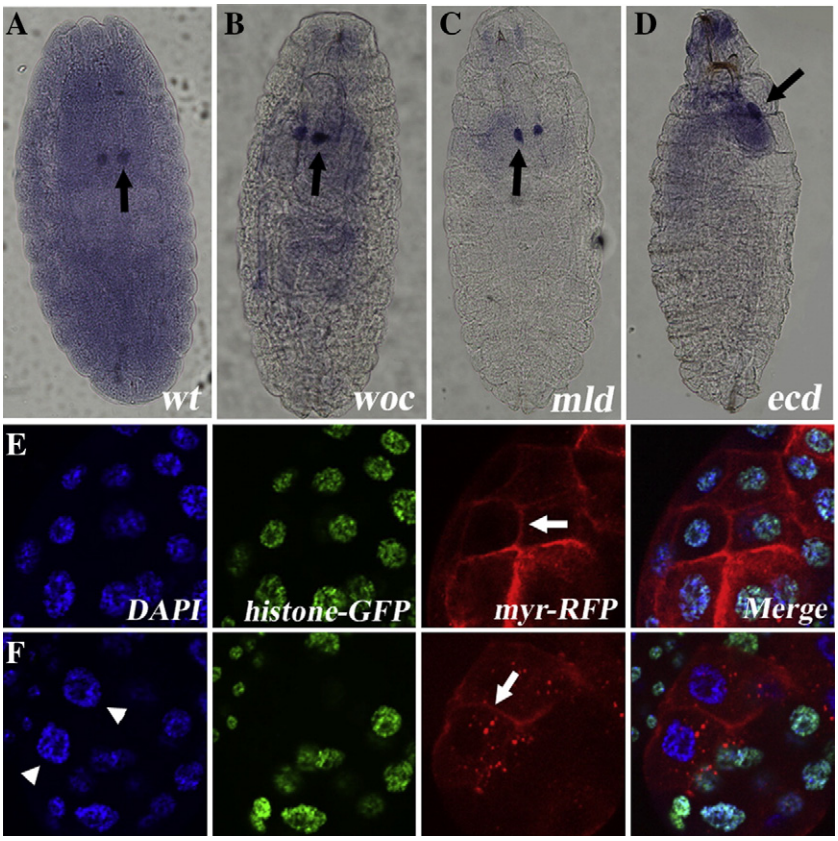


Fig. 1. *woc*, *mld*, *ecd*, and *ftz-f1* do not regulate *npc1* ring gland expression. (A–D) *In situ* hybridization showing *npc1* signal detection in the wild type and in *woc*, *mld*, and *ecd* mutants. Anterior is up. Arrows indicate the ring glands. (E–F) *npc1-Gal4* drives *UAS-myr-RFP* expression in *ftz-f1* ring gland clones. Nuclei are labeled with DAPI. Nuclear GFP signals highlight wild type and *ftz-f1*/+ cells. The area with no GFP expression is the *ftz-f1* mutant clone (arrowheads in F). Arrows show myr-RFP expression. (E) myr-RFP expression in the wild type. (F) myr-RFP expression is normal in the *ftz-f1* mutant clone. Arrowheads show the *ftz-f1* mutant clone.

using a 2.3-kb *npc1* promoter including a 300-bp upstream region, the first exon (encoding the UTR), and the first intron. The *Gal4* transgene drove strong *UAS-GFP* expression in ring glands, indicating that the ring gland-specific *cis*-regulatory elements are within this 2.3-kb region (Figs. 2A and B). A series of Gal4 transgenes with truncations of the 2.3-kb promoter were made. A 220-bp *npc1* promoter element when fused to *Gal4* with an *hsp70* minimal promoter was found to be sufficient to drive *UAS-GFP* ring gland-specific expression (Figs. 2A and B). Thus, we named it the ring gland-specific *cis*-regulatory element (RSE). The RSE region is right after the first exon of *npc1* which consists of part of the 5′ UTR (Fig. 2A).

To determine the *in vivo* function of RSE, we turned to examine the expression of *npc1* in RSE-deleted *npc1* mutants. $npc1^{27A}$ is a small deletion including the 220-bp RSE region (Fig. 3A) (Fluegel et al., 2006; Huang et al., 2005). Since $npc1^{27A}$ also affects the transcription of the upstream gene *Pros35* and is first instar larval lethal, we examined the expression levels of *npc1* in the ring gland of $npc1^{2}/npc1^{27A}$ *trans*-heterozygous mutants. $npc1^{2}$ bears a large deletion spanning the first exon to the fifth exon and is a null allele of *npc1* (Fig. 3A). We found that *npc1* transcription was greatly reduced, but not totally diminished, in the ring gland of $npc1^{2}/npc1^{27A}$ animals in several different assays including *in situ* hybridization, RT–PCR, and quantitative RT–PCR (qRT-PCR) (Figs. 3B–D). Although we cannot exclude the possibility that other regions besides the 220-bp RSE in the $npc1^{27A}$-deleted region may contribute to the results, these results support the notion that the RSE is likely the ring gland-specific enhancer for *npc1 in vivo*. Interestingly, these results appear slightly difference from the clear-cut GFP on/off results in the *UAS-Gal4* assay (Fig. 2), suggesting that besides RSE some unidentified basal regulatory regions may exist in the *npc1* gene. Therefore, without the RSE, *npc1* is expressed at basal level. The residual expression of *npc1* in $npc1^{2}/npc1^{27A}$ mutants also explains the larval/pupal lethal phenotype observed in *trans*-heterozygous mutants and is consistent with previous findings that the *npc1* null mutants are first instar larval lethal (Huang et al., 2005).

The RSE is conserved in Drosophila

We next sought to identify the key DNA sequence within the 220-bp region. We first took an evolutionary approach. We cloned the RSE region from five other *Drosophila* species in the *melanogaster* group, namely *simulans*, *sechellia*, *yakuba*, *erecta*, and *ananassae*. Transgenes harboring *RSE-Gal4* from various species were made in *D. melanogaster*. We tested Gal4 activity by crossing them to *UAS-GFP* flies. All the transgenes could drive GFP expression strongly in the ring gland, indicating that the RSE element is conserved throughout evolution in the *melanogaster* group (Fig. 4A).

The RSE DNA sequences from different *Drosophila* species were compared using the Vista Genome Browser and DNA alignment (Figs. 4B and C). Two highly conserved motifs, each about 25 bp in length, were found. Specifically, the second conserved motif contains a 20-bp sequence, which is identical in all six species. To determine the importance of these two conserved motifs, we generated several *Gal* transgenes with deletion in either motifs and found that they abolished the ring gland-specific expression in the *UAS-GFP* reporter assay (Fig. 4D). Therefore, we concluded that the conserved sequence in the RSE is critical for *npc1* ring gland-specific expression.

The RSE is regulated by br

We then searched for potential transcription factor binding sites in the 220-bp sequence using the TransFac program (www.gene-

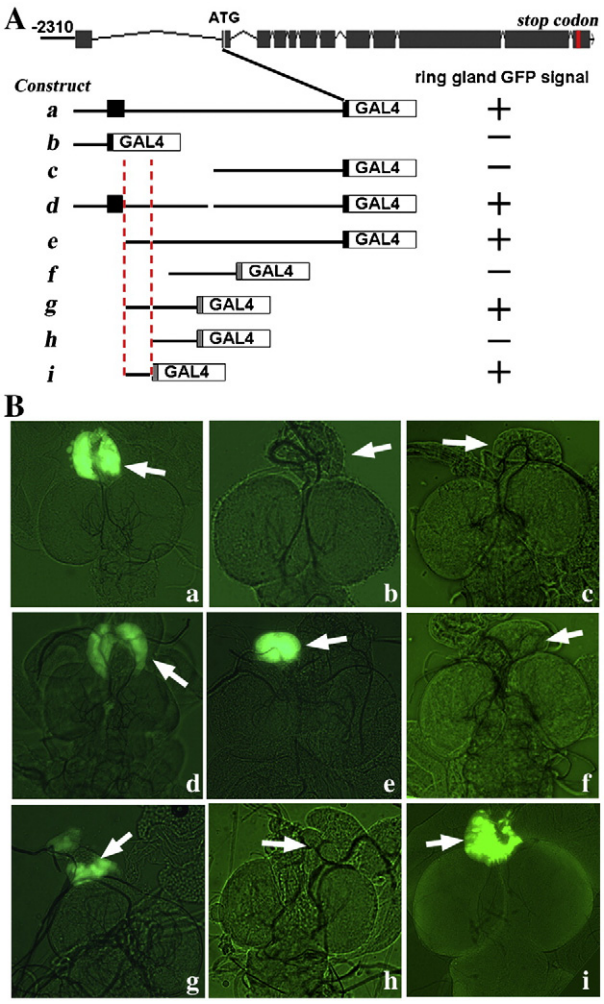


Fig. 2. A 220-bp region is important for *npc1* ring gland-specific expression. (A) A series of *npc1* promoter *Gal4* fusion constructs were made. The first five constructs are in a *pC3G4* vector background. The last four constructs are in a *pPT-GAL* background, which contains an *hsp70* minimal promoter. The transgenes were crossed to *UAS-GFP* lines to observe the GFP expression pattern in their progeny. "+" means positive ring gland GFP signal; "−" shows negative ring gland GFP. The region between the two red dashed lines is the 220-bp RSE region. (B) Positive and negative ring gland GFP expressions in the *UAS-Gal4* assay for transgenes with constructs described in (A). The arrows indicate the ring glands.

regulation.com). The transcription factor BR stood out. *br*, also known as *broad* or *broad complex* is a well-known ecdysone early response gene and functions in the regulation of developmental processes, including the morphogenesis of imaginal discs, metamorphosis, and pupariation (Fletcher and Thummel, 1995; von Kalm et al., 1994). The *br* locus is a complicated locus, and mutants in the *br* locus can be classified into several different complementation groups (Bayer et al., 1997; Crossgrove et al., 1996). Four groups of fully complementing alleles [*br*, *rbp*, *l(1)2Bc*, and *l(1)2Bd*] of *br* were identified. In addition, there are noncomplementing alleles (*npr* for non-pupariating), which fail to complement all these four groups, suggesting that they are putative null alleles of *br*. br^{npr} mutants are arrested as third instar larvae and eventually die after several days as wandering third instar larvae without metamorphosis. Although br^{npr} mutants are able to produce ecdysone, implantation of wild-type ring gland can partially rescue br^{npr-3} mutants, suggesting that br^{npr-3} may have impaired ecdysone biosynthesis (Kiss et al., 1980). BR has two functional domains, a protein–protein interacting BTB domain and a DNA-binding Zinc finger domain. There are four isoforms (Z1–Z4) of BR, and they share the same BTB domain with different C2H2 Zinc finger domains. The Zinc finger domain thus likely determines the target specificity. BR-Z4 was predicted to bind to a TGTTTAC sequence, which is present in the second conserved motif in the RSE (Fig. 4C) (von Kalm et al., 1994). Deletion of this seven nucleotide sequence or point mutations of the central TTT nucleotides all totally abolished the ring gland-specific expressing activity in *UAS-Gal4* assay, indicating that indeed the predicted binding site for BR-Z4 is essential for RSE function (Fig. 4D).

Does BR-Z4 regulate *npc1* expression *in vivo*? Results from RT–PCR, qRT-PCR and *in situ* hybridization are consistent with the conclusion that BR regulates *npc1* ring gland-specific expression. In br^{npr-3} mutants, only basal expression of *npc1* is detected, which is consistent with the larval/pupal lethal phenotype in br^{npr-3} mutants (Figs. 5A–C). These findings are also in agreement with the lethal stage of RSE-deleted $npc1^{2}/npc1^{27A}$ *trans*-heterozygous mutants and the residual *npc1* expression in RSE-deleted *npc1* mutants (Figs. 3B–D).

The above experimental results suggested that BR is essential for *npc1* ring gland-specific expression and that the RSE is a ring gland-specific *cis*-regulatory element. To further explore the connection between *br* and *npc1*, we took advantage of heat-inducible *br* transgenes and examined *npc1* transcription levels in response to heat (Zhou et al., 2004). Upon heat shock treatment, the transcription levels of *npc1* increased in concert with the elevation of *br* in a heat-shock-responsive *br-Z4* transgene (Fig. 5D). In contrast, the same treatment to the heat-shock-responsive *br-Z1*, *-Z2*, or *-Z3* transgenes did not result in the same enhancement pattern (Fig. 5D). Consistently, in br^{npr-3} mutants, we found an arginine-to-cystine mutation within the C2H2 zinc finger domain as well as a small in-frame deletion in Br-Z4 isoform (Fig. S1). The above results suggest that BR-Z4 likely positively regulates *npc1* ring gland-specific expression. However, it also raises another question. Since BR is broadly expressed in many larval tissues (Emery et al., 1994), why is *npc1*

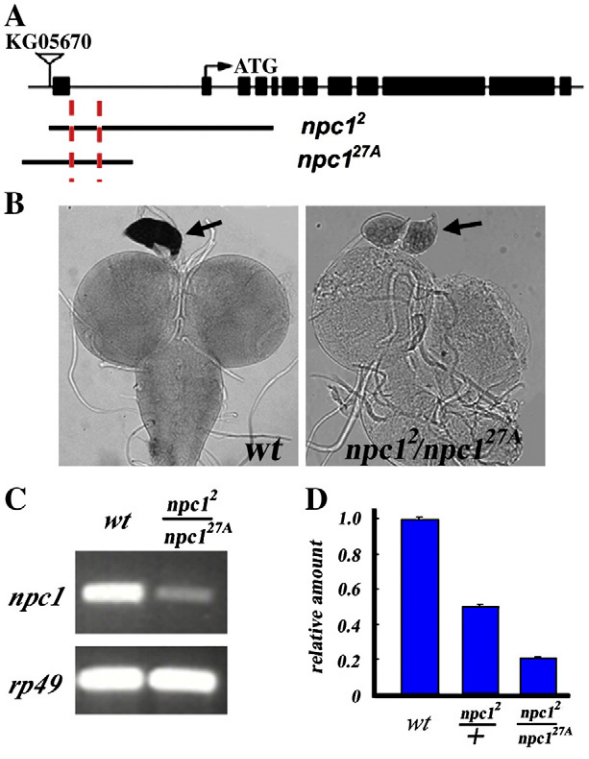


Fig. 3. *npc1* ring gland expression is decreased in RSE-deleted *npc1* mutants. (A) Two alleles of *npc1*. The region within the two red dashed lines is the RSE. (B) *In situ* hybridization shows the *npc1* expression level in the ring glands (arrow) of wild type and $npc1^{2}/npc1^{27A}$ third instar larvae. (C) RT-PCR shows that the *npc1* expression level is greatly reduced in $npc1^{2}/npc1^{27A}$, (D) Compared to $npc1^{2}/+$, the *npc1* expression level in ring gland is reduced ~60% in $npc1^{2}/npc1^{27A}$ *trans*-heterozygotes by qRT–PCR. Error bars represent the standard error of the mean (SEM).

highly expressed in the ring gland? A possible explanation is that there are other ring gland-specific factor(s) acting together with BR in regulating the ring gland-specific expression of *npc1*.

BR may bind to npc1 RSE with the help of other protein(s)

Does BR regulate *npc1* ring gland-specific expression directly or indirectly? We used EMSA to examine the physical interaction between BR and the BR-Z4 binding sequence of the RSE (RSE core). Recombinant GST-fusion proteins of full-length BR-Z4 and the DNA binding domain of BR-Z4 were made. However, when incubating with the RSE core probe, neither protein could result in shift band in nondenatured PAGE gels (data not shown). At least three possibilities may explain these results. First, the recombinant proteins did not fold correctly and were not functional. Second, BR protein does not bind to RSE directly. Third, the binding of BR to RSE may require the help of other protein(s). Based on results from the above reporter analysis and the expression patterns of *br* and *npc1*, we next investigated the last possibility.

We firstly prepared whole embryo/larval nuclear extracts and tested their binding to the RSE core probe. Nuclear extracts from wild-type embryo/larvae led to a high-molecular-weight shift of the wild-type RSE probe, suggesting that the RSE probe could bind to protein(s) in the nuclear extract (Fig. 6A). Moreover, under extended exposure time, two low-molecular-weight shift bands appeared (Fig. 6B, lane 1). All these shifted bands disappeared when competing with cold RSE core probe, indicating that binding was specific. In addition, the shift did not take place with the BR consensus binding site-mutated RSE core probe incubated with the nuclear extract, further supporting binding specificity (Fig. 6A). To test whether any of these shifted bands are directly associated with BR, we then prepared nuclear extracts from *br* mutant larvae. The high-molecular-weight shift band persisted, indicating that there are other protein(s) in the *br* mutant extract which can bind to the RSE core probe. However, interestingly, the low-molecular-weight shift bands were greatly diminished or absent in the assay, suggesting that BR might be crucial for these mobility shifts (Fig. 6B, lane 2). To further prove this idea, we added GST-BR-Z4 into the nuclear extracts of wild type and *br* mutants. We found that adding GST-BR-Z4 into the *br* mutant extracts restored the two low-molecular-weight shift bands, while adding the GST control did not have such an effect (Fig. 6B, lanes 3–5 and 7). In addition, adding GST-BR-Z4 to the wild-type extract also did not affect the mobility shift. These results suggest that BR protein is vital for the formation of low-molecular-weight shift bands with the RSE core probe. Moreover, this echoes the previous hint that other factor(s) may act together with BR in binding to the RSE of *npc1* to regulate *npc1* ring gland-specific expression.

BR is a general regulator for ecdysone biosynthesis

Since $br^{npr\text{-}3}$ mutants are larval/pupal lethal with reduced *npc1* ring gland-specific expression, we examined whether increasing *npc1* expression could rescue *br* null mutants. Both ring gland-specific *Gal4* and ubiquitous *Gal4* driven *UAS-npc1* did not rescue the larval arrest phenotype of $br^{npr\text{-}3}$, indicating that compensating *npc1* expression is not sufficient to rescue *br* (data not shown). It further implies that *npc1* is likely not the solo target of *br* in the ring gland and that there may be other targets of *br*. Previous studies implied that $br^{npr\text{-}3}$ mutants are likely to be defective in ecdysone biosynthesis (Kiss et al., 1980). Therefore, we examined a battery of ecdysone biosynthesis or ring gland development-related genes. The BR-Z4 binding motif is found in the promoter region of these ecdysone biosynthesis-related genes (Fig. 7A). We then examined the transcription levels of these genes in ring glands of $br^{npr\text{-}3}$ mutants by qRT–PCR. Similar to *npc1*, the transcription levels of 8 out of 10 genes, including *ecd*, *mld*, *start1*, and five ecdysone biosynthetic enzyme genes, were greatly reduced in $br^{npr\text{-}3}$ mutants (Fig. 7B). In contrast, *woc* transcription was increased, while there was no change in *giant* transcription. *giant* encodes RNA polymerase II, which is required for ring gland development (De Velasco et al., 2004). Therefore, *br* seems to act as a general regulator for a set of ecdysone biosynthesis-related genes in the ring gland.

To further prove that *br* acts in the ring gland to regulate the expression of these ecdysone biosynthesis-related genes, we knocked down *br* specifically in the ring gland by crossing *UAS-br-RNAi* transgene with a ring gland-specific *Gal4*, *P0206* (Mirth et al., 2005). We obtained similar results, indicating that indeed *br* could function in the ring gland to regulate the expressions of ecdysone biosynthesis-related genes (Fig. 7C). Moreover, ring gland-specific knockdown of *br* led to third instar larval-arrest animals, and most of which cannot leave the food and wander up to the wall to form pupae. It is well known that this wandering behavior is dependent on ecdysone (Dominick and Truman, 1985). Indeed, the wandering phenotype in the RNAi animals could be partly rescued by ecdysone (2/20 wandering-up in control animals versus 17/20 in 40 μg/ml ecdysone-treated animals), suggesting that ring gland-specific knockdown of *br* results in ecdysone deficiency. However, ecdysone cannot rescue the wandering-defective animals to late pupae or adult, indicating that there are other ecdysone-independent defects associated with ring gland-specific knock down of *br*.

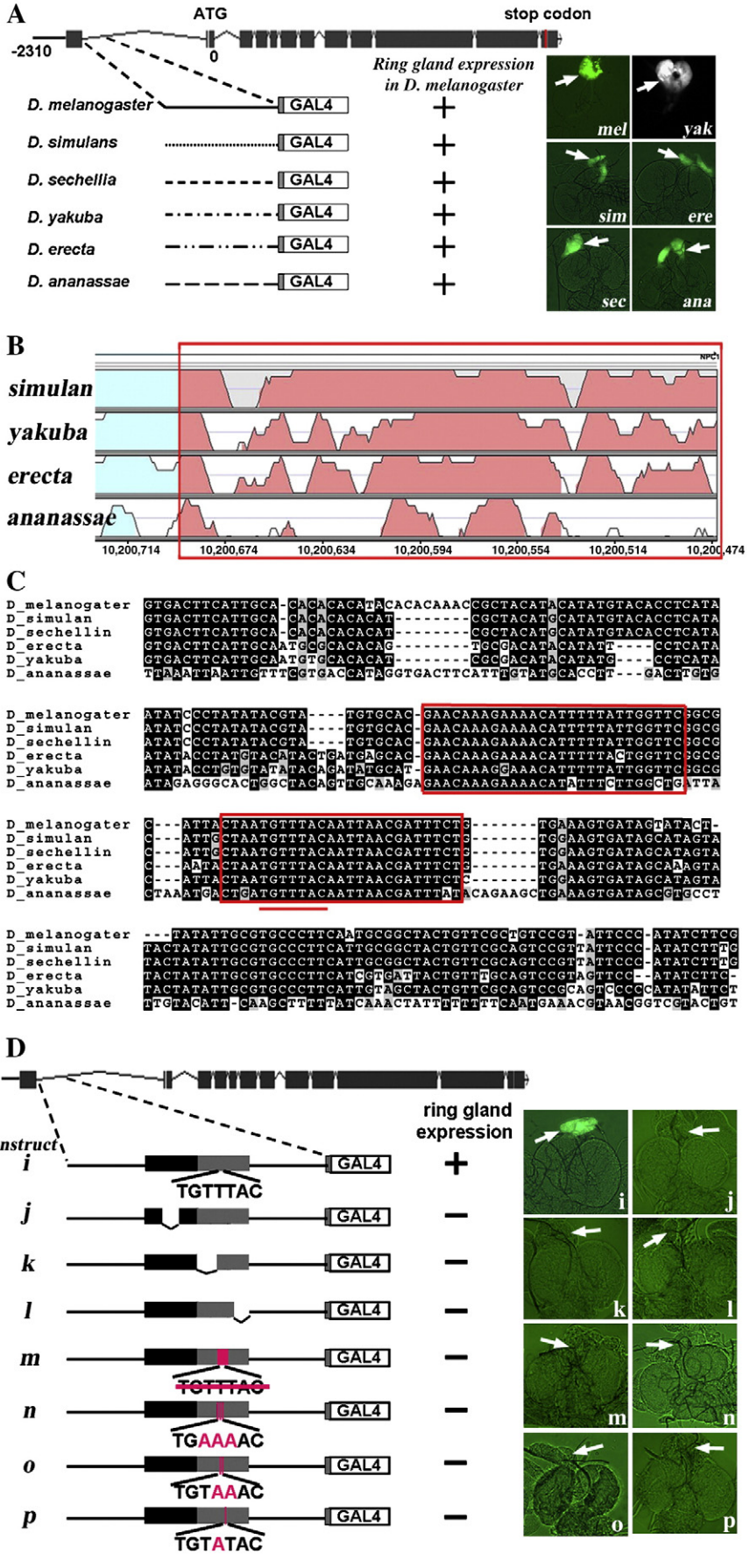
PTTH-Torso and insulin pathways were also reported to be important for ecdysone biosynthesis (Colombani et al., 2005; Rewitz et al., 2009). Could *br* affect these two pathways as well? We found that the expressions of both *torso* and *InR* are greatly reduced in the ring glands of $br^{npr\text{-}3}$ mutants and ring gland-specific *br* RNAi animals (Figs. 7B and C), suggesting that BR may regulate the transcription of ecdysone biosynthesis genes directly, or alternatively, indirectly through these two pathways.

Discussion

Ecdysone hormone produced in the ring gland plays a central role in regulating insect development. In this study, we have identified RSE, a ring gland-specific *cis*-regulatory element, in the promoter of the ecdysone biosynthesis-related gene *npc1*. In addition, *br*, an ecdysone early response gene, was found to be a key regulator for the ring gland expression of *npc1*. Moreover, *br* seems to regulate the expression of many other ecdysone biosynthesis-related genes in the ring gland.

The functions of *br* have been studied extensively. As an ecdysone early response gene, it regulates the transcription of many late response genes, including *L71*, *sgs-4*, and *hsp23*, in response to the ecdysone signal (Dubrovsky et al., 1996, 2001; von Kalm et al., 1994). *br* also regulates the expression of *Drosophila* caspase Dronc, which is important for programmed cell death during metamorphosis (Cakouros et al., 2002; Daish et al., 2003). The null allele of *br*, $br^{npr\text{-}3}$ displays an ecdysone-deficient phenotype, arresting at wandering third instar for a long time, which is consistent with its role in mediating the ecdysone response. However, it was found that implanting a wild-type ring gland can partially rescue the $br^{npr\text{-}3}$ phenotype, implying that *br* mutants may be partial ecdysone

Fig. 4. RSE is conserved in other *Drosophila* species. (A) RSEs from five other *Drosophila* species are functional in *Drosophila melanogaster*. The arrows indicate the ring glands. (B) RSE sequence alignment using the VISTA Genome Browser. The RSE sequences (boxed in red) from the four other *Drosophila* species (*simulan*, *yakuba*, *erecta*, and *ananassae*) were compared to the RSE from *Drosophila melanogaster*. The conserved regions are filled with red. (C) Sequence alignment of RSE. Two red boxes mark two conserved domains in RSE. The red line highlights the predicted BR-Z4 binding sites (RSE core). (D) Deletion or point mutations of BR-Z4 binding sites impair RSE function. Grey box and the black box: the first and the second conserved domains in RSE. "+" means positive ring gland GFP signal; "−" shows negative ring gland GFP in the *UAS-Gal4* assay. The arrows indicate the ring glands.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Ring gland expression in D. melanogaster
D. melanogaster
D. simulans
D. sechellia
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D. erecta
D. ananassae
GAL4
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yakuba
erecta
ananassae
10,200,714
10,200,674
10,200,634
10,200,594
10,200,554
10,200,514
10,200,474
C
D_melanogater
D_simulan
D_sechellin
D_erecta
D_yakuba
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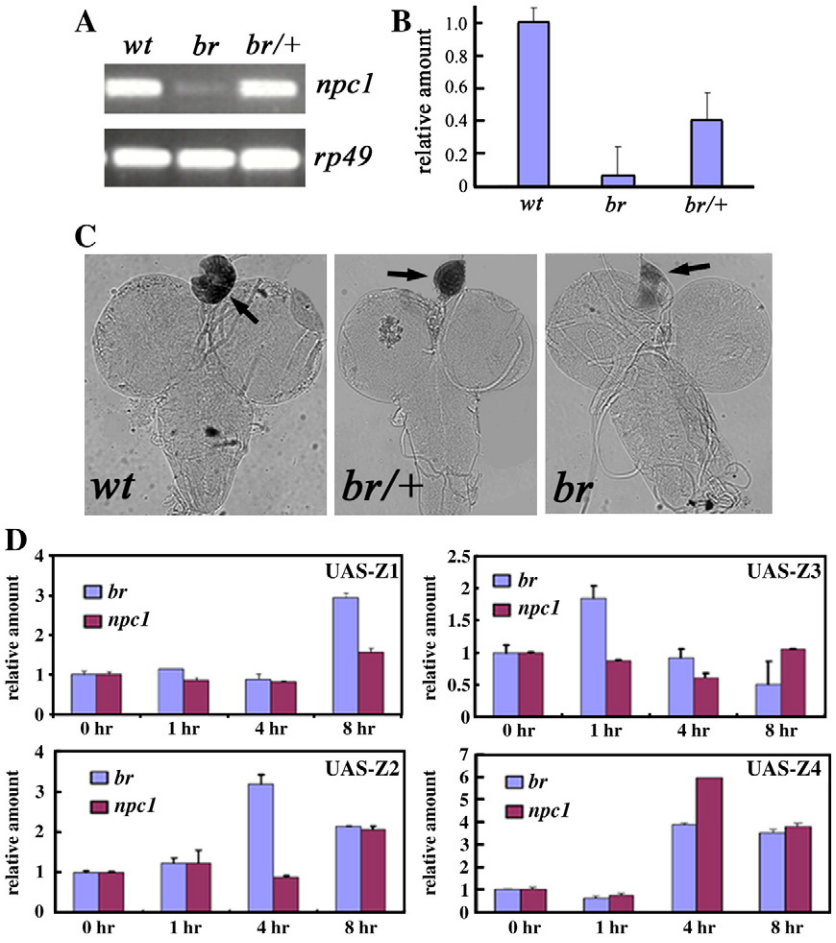

Fig. 5. *br* is necessary for *npc1* ring gland expression. (A) RT–PCR shows that the *npc1* expression level decreased in the ring gland of *br* mutants. (B) Decreased *npc1* ring gland expression in *br* mutants measured by qRT–PCR. Error bars are the SEM. (C) *In situ* hybridization shows that *npc1* is highly expressed in the wild-type ring gland and that the expression is greatly reduced in *br* mutants. The arrows show the ring gland. (D) The changes in *npc1* levels show similar patterns to those of *br-Z4* but not other *br* isoforms when the expressions of different isoforms of *br* were heat-shock-induced individually.

deficient (Kiss et al., 1980). Previous studies on the role of BR have mainly focused on ecdysone-responding tissues, for instance the salivary gland (Guay and Guild, 1991). It remains unclear whether BR also affects ecdysone biosynthesis. Our findings suggest that besides having a role in triggering ecdysone late response gene expression in larval peripheral tissues, *br* also has an important role in regulating ecdysone biosynthesis in the ecdysone-producing organ. In addition, the fact that the larval-arrest phenotype in the animals with ring gland-specific knockdown of *br* was only partially rescued by ecdysone indicates that *br* may have other roles in the ring gland. In agreement with that, experiments with ring gland-specific over-expression of different *br* isoforms revealed that *br* may function in regulating the degeneration of ring gland (Zhou et al., 2004).

Our study implies the existence of a positive feedback loop in which ecdysone regulates the transcriptional expression of the early response gene *br* and *br* could subsequently augment the transcription of ecdysone biosynthesis-related genes to further boost ecdysone production in the ring gland. The feedback regulation of ecdysone biosynthesis has been well documented in insects and ecdysone could have both positive and negative roles on ecdysone biosynthesis (Gilbert et al., 2002; Marchal et al., 2010). What is the significance of such a positive feedback regulation mechanism? During development, ecdysone levels increase and decrease rapidly before molt and after molt, respectively, as well as before pupariation and after pupariation (Gilbert et al., 2002). The mechanism by which such quick changes of ecdysone levels are achieved remains elusive. We believe that the positive feedback regulation may facilitate the rapid increase in ecdysone biosynthesis. On the other hand, this feedback may be involved in the subsequent decline in ecdysone biosynthesis after molt or pupariation. Therefore, this feedback regulation could help to fine-tune ecdysone biosynthesis within a small time window during rapid development.

Our initial aim was to identify a ring gland-specific factor that acts on the RSE to regulate *npc1* tissue-specific gene expression. However, several known ring gland-specific transcription regulators seem not to be required for *npc1* expression. In contrast, BR, which is not a ring gland-specific protein, was proved to be vital for *npc1* ring gland expression. Our studies suggest that there are unknown factor(s) involved in *npc1* transcriptional regulation. Wild-type nuclear extracts led to a high-molecular-weight shift of the RSE core probe, suggesting that these factors may form a large protein complex. Unfortunately, the identity of these unknown factors remains to be determined. More work, for example purifying BR interacting proteins to reveal their identity and to examine whether they are ring gland-specifically expressed, needs to be done.

The RSE identified in this study is the first ring gland-specific element to be discovered. It is conserved through evolution in several *Drosophila* species. RSEs of other *Drosophila* species are active in *D. melanogaster*. Consistently, the regulator BR is also conserved (Spokony and Restifo, 2007). Moreover, the RSE core BR-Z4 binding site is present in a set of ecdysone biosynthesis genes, suggesting that the RSE is important for ecdysone biosynthesis. In addition, many ring gland-specific *Gal4* lines have previously been reported, including *2-286*, *P0206*, *May60*, and *phm* (Fluegel et al., 2006; Huang et al., 2005;

McBrayer et al., 2007). Besides *phm-Gal4*, the regulatory sequences for these *Gal4* lines are unknown. We found that while the activity of *npc1-Gal4* is regulated by *br*, the activity of *P0206-Gal4* is also regulated by *br* (data not shown). On the other hand, the RSE is likely not the only ring gland-specific element. There are other ring gland-specific transcription regulators, such as *ecd*, *mld*, and *woc*, which do not act on the RSE of *npc1*. The targeting regulatory elements for these genes have not yet been identified. Finding such elements would undoubtedly advance our knowledge of the regulation of ecdysone biosynthesis.

As an evolutionary conserved gene, *npc1* is important for cholesterol trafficking in many other systems including mammals. In mice, *npc1* is vital for neurosteroid biosynthesis, which is likely a key factor determining the neurodegenerative phenotype of *npc1* mutants (Griffin et al., 2004). Our studies on the regulation of *Drosophila npc1* tissue-specific expression by *br* may contribute to studies on the regulation of neurosteroid biosynthesis in higher animals.

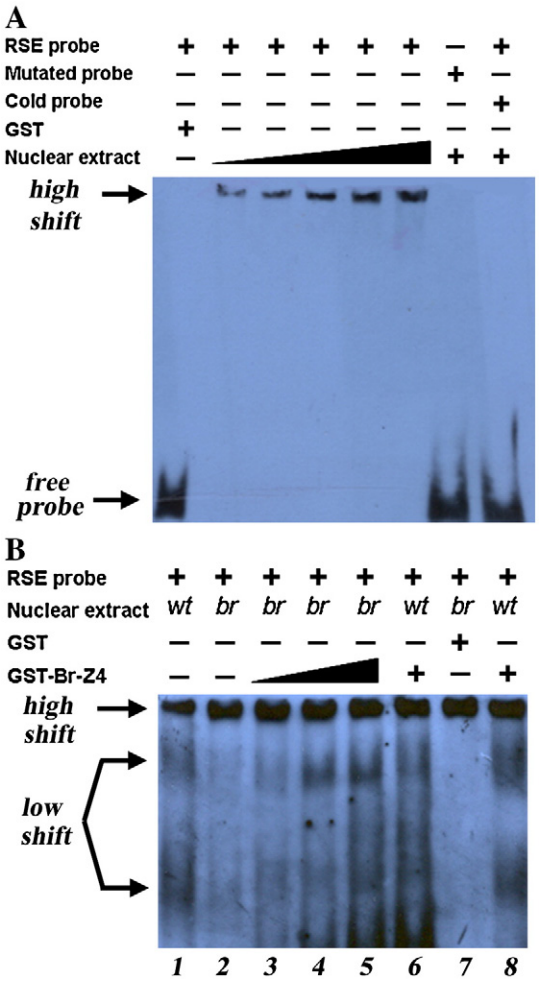

Fig. 6. BR likely binds to *npc1* RSE with the help of other protein(s). (A) The nuclear extract from wild-type embryos can bind to the RSE core probe but not to the mutated probe. High-molecular-weight shift bands are indicated. Equal amounts (0.2 ng) of both wild-type and mutated probes were used. From left to right: lane 1 = 40 μg of GST protein; lanes 2–6 = 3, 6, 12, 24, and 40 μg of nuclear extract, respectively; lane 7 = 24 μg of nuclear extract; lane 8 = 24 μg of nuclear extract and 500× nonlabeled wild-type probe. (B) BR likely binds to the RSE probe forming low-molecular-weight shifts with the help of other protein(s). Both high- and low-molecular-weight shift bands are indicated. From left to right: lane 1 = 25 μg of nuclear extract from wild-type larvae; lanes 2–5 = 25 μg of nuclear extract from *br* mutant larvae; lanes 3–5 = 4, 8, and 15 μg of GST-BR-Z4 protein, respectively; lane 6 = 25 μg of nuclear extract from wild-type larvae and 15 μg of GST-BR-Z4 protein; lane 7 = 25 μg of nuclear extract from *br* mutant larvae and 15 μg of GST protein; lane 8 = 25 μg of nuclear extract from wild-type embryo and 15 μg of GST-BR-Z4 protein.

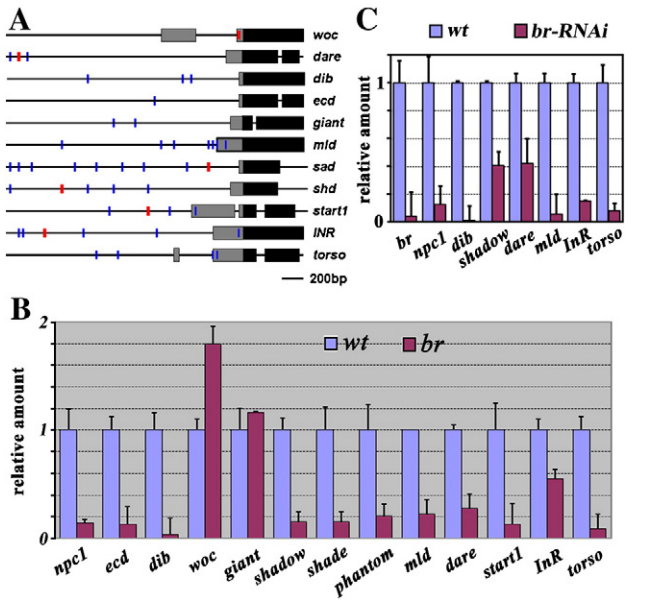

Fig. 7. *br* also regulates the ring gland expression of many ecdysone biosynthesis-related genes. (A) The Br-Z4 binding site is present in many ecdysone biosynthesis-related genes. For each gene, a 2-kb region upstream of start codon ATG was used for binding site search. Grey box: UTR; black box: protein coding region. Blue bar: putative Br-Z4 binding site; red bar: putative Br-Z4 binding site which is conserved in six species of the *melanogaster* group. (B) qRT–PCR of the transcripts of *npc1*, *ecd*, *dib*, *woc*, *giant*, *shadow*, *shade*, *phantom*, *mld*, *dare*, *start1*, *torso*, and *InR* in the ring gland of the *br* mutants. Error bars represent the standard error of the mean (SEM). (C) qRT–PCR of the transcripts of *npc1*, *dib*, *shadow*, *mld*, *dare*, *torso*, and *InR* in the ring gland of the ring gland-specific *br* RNAi animals. Error bars represent the standard error of the mean (SEM).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.05.510.

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